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CONSTRUCTION AND CHARACTERIZATION OF RANDOM MUTANT LIBRARY USING Tn5 TRANSPOSOME IN *MESORHIZOBIUM CICERI* Ca181

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ABSTRACT

Mesorhizobium ciceri is a nitrogen fixing bacterium which forms symbiotic root nodule association with chickpea plant. After completion of *M. ciceri* genome sequencing, functional characterization of the predicted genes has become feasible to help strain improvement for better bio-fertilizers. The aim of present study was to create a transposon insertion mutant library of *M. ciceri* strain Ca181 using commercially available Tn5 EZ: :Tn5 <Kan-2> Tnp transposome system from Epicentre. The Tn5 random mutagenesis system used in the present study is more efficient than previously reported *M. ciceri* random mutagenesis approaches and an efficiency of 0.6×10^6 cfu μg^{-1} transposome was observed. The method was validated by studying auxotrophy of the mutants. One auxotrophic mutant was identified for proline biosynthesis after screening of 576 random mutants, and the disrupted genomic region was identified by inverse PCR and subsequent sequencing of the transposon flanking regions. This is the first transposon knockout population reported for *M. ciceri* using electroporation technique.

Abbreviations: IPCR, Inverse PCR; VMM, Vincent Minimal Medium; YEMA, Yeast Extract Mannitol Agar

KEYWORDS: Tn5 Mutagenesis, *Mesorhizobium*, Mutant Library, Transposome

INTRODUCTION

Although about eighty percent of the air is nitrogen Crop plants are unable to utilize this for their nutrition and huge quantity of nitrogenous fertilizers are applied to the soil for realizing high crop productivity, particularly in cereals. *Mesorhizobium ciceri* is a gram negative bacterium which forms nitrogen fixing nodules in the roots of chickpea plants (*Cicer arietinum*). The formation of bacterial root nodules occurs in specialized cells due to symbiotic associations between rhizobia and leguminous plants [1]. Decoding of the complete genome sequence of *M. ciceri* would help better understanding of the mechanisms of nitrogen fixation, abiotic stress tolerance and competitiveness of different rhizobia strains in the soil mutually beneficial interaction with the host plant. Transposon mutagenesis is a powerful tool for functional genomic studies of *M. ciceri*. Transformation by means of conjugation reported in earlier studies, requires laborious selection procedures to purify the transformants from donor bacteria [2] The Tn5 based transposome system has been described by Goryshin *et al.* [3]. The transposable element of the transposome contains a kanamycin resistance gene, flanked on both sides by 19 bp inverted repeats of the Tn5 transposon. This transposon element is complexed with hyperactive transposase protein which forms a functional transposome.This functional transposome can easily be transferred to bacterial cells by electroporation. The transposome system has been used widely in many bacteria for creation of gene knock out mutant libraries, e.g. *Pseudomonas* [4], *Xanthomonas* [5] and *Gluconoacetobacter* [2]. However, so far use of transposome system has not been reported for the chickpea rhizobium *M. ciceri*.

In the present study, a protocol was standardized to generate large number of random genetically stable insertional mutants of *M. ciceri* Ca181 by electroporation using Tn5 transposon EZ: : Tn5 <Kan-2> Tnp transposome. The exact insertion site of the transposon in an auxotrophic mutant for proline synthesis was identified by inverse PCR (IPCR) followed by sequencing of the flanking regions.

MATERIALS AND METHODS

The wild type *M. ciceri* strain Ca181 forms nodules and fixes atmospheric nitrogen in the roots of chickpea plant. The original strain was obtained from ICRISAT, Hyderabad. It is grown routinely at 28°C on YMB (Yeast Extract Mannitol broth) or in Vincent minimal medium [6]. The broad host range vector pBBR122 (Mobitech) containing Chloramphenicol gene was used to test the electroporation efficiencies. Tn5 mutants were screened on plates containing nalidixic acid (10 µg ml^{-1}) and kanamycin (50 µg ml^{-1}).

Two ml of a stationary phase culture was used to inoculate 250 ml of YEM broth in a 500 ml flask. The culture was incubated at 28 °C with vigorous shaking for 24 h until it reached mid logarithmic phase (OD_{600} of 0.4-0.6). Cells were chilled on ice for half an hour and then harvested by centrifugation at 5000 rpm for 15 min at 4 °C. The cell pellet was washed three times with 25 ml cold sterile 300 mM sucrose solution and finally washed and resuspended in 0.2ml of 300 mM sucrose solution supplemented with 15% glycerol. One hundred microlitres of the resulting suspension was aliqouted in ice-cold 1.5 ml polypropylene microcentrifuge tubes, snap freezed in liquid nitrogen and stored at -80 °C to be used later as electro competent cells.

To test the transformation efficiency of the electro competent *Mesorhizobium* cells, 10 ng of pBBR122 vector DNA was mixed with 50 µl of electrocompetent cells in a 0.1 cm electrode gap electroporation cuvette from Biorad. For pulse generation, a multiporator (Eppendorf) was used that was capable of generating a field strength of up to 18 kVm^{-1} with a 0.1 cm gap cuvette. Electroporation was carried out at different voltages (900 V-1800 V) for 5 ms in order to find optimum voltage for maximum transformation efficiency. After electroporation 1 ml of YEM broth was immediately added to the cell suspension and incubated for 24 h with vigorous shaking at 28 °C. The cell suspension was diluted and plated on YEMA supplemented with 10 µg $µl^{-1}$ of cholramphenicol. The numbers of CFU were scored after 6 days of incubation at 28 °C. Two *M.ciceri* colonies harbouring pBBR122 were further tested for plasmid stability. The colonies were grown in selective medium at 28 °C for 6 days with shaking. Successive subcultures were established using 100 µl of the initial culture to 10 ml of YEM broth (this was done in triplicates) for 18 days. The samples were taken after every six days and dilutions were plated on YEMA plates. Total 96 colonies from each time point were tested for chloramphenicol resistance. The transformants generated with pBBR122 were highly stable as no loss of resistance to chloramphenicol was observed when cells were grown without selection.

Transposon insertion mutagenesis was carried out using 20 ng of EZ::TN<KAN-2> transposome into *M. ciceri* Ca181 by electroporation. The electroporation mix was incubated with 1 ml of YEM broth for 24 h with shaking at 28 °C and then spread on YEM agar plates supplemented with nalidixic acid and kanamycin. Mutants which were defective in amino acid biosynthesis were identified by cross-pool auxanography method described by Holliday [7] on VMM with or without nutritional supplement.

The selected mutants were grown individually and total genomic DNA was isolated using Wizard genomic DNA isolation kit (Promega). Presence of Tn5 within the chromosomal DNA was confirmed by amplifying a 800 bp product using primers specific to the Tn5 sequence: KaninF 5'GGTGGACCAGTTGGTGATTT 3' and KaninR 5'ATTCAACGGGAAACGTCTTG 3'. PCR cycling conditions were: initial denaturation at 95 °C for 5 min; 30 cycles of

denaturation at 95 °C for 30 s, annealing at 55 °C for 20 s and extension at 72 °C for 2 min; final extension at 72 °C for 10 min in an Eppendorf master cycler. The PCR products were examined via standard agarose gel electrophoresis and sequenced using specific primers for Tn5.

Transposon mutants of *M. ciceri* Ca181 were mapped for transposon insertions site by adapting inverse PCR as described by Fernandes et al [8]. One to two µg of DNA samples from mutant was digested with appropriate enzymes (*EcoR*V, *Kpn*I and *Nco*I). The digested DNA was self-ligated and IPCR was performed using outward primers from transposon (KAN-2 FP-1 5' ACCTACAACAAAGCTCTCATCAACC 3' and KAN-2 RP-1 5' GCAATGTAACATCAGAGATTTTGAG 3'. PCR cycling conditions were as follows : 95 °C for 5 min; 30 cycles of 95 °C for 30 s, 55 °C for 20 s, 72 °C for 2 min and final extension at 72 °C for 10 min using Eppendorf master cycler. Inverse PCR product was gel purified using Qiagen Gel Extraction kit (Qiagen), cloned into pGEMT Easy Cloning Vector (Promega) and transformed into *E. coli* DH5α. The recombinant plasmid was then sequenced using M13 forward primer on an ABI DNA analyser platform. *M. ciceri* sequences flanking the kanamycin containing transposon were determined using the nucleotide BLAST programme of National Centre for Biotechnology Information (http://blast.ncbi.nlm.nih.gov).

RESULTS AND DISCUSSIONS

Wild type *M. ciceri* Ca181 was unable to grow on plates 10µg ml^{-1} of kanamycin and chloramphenicol when incubated at 28°C for 7 days in all replicates. Transformants obtained through transposon mutagenesis grew at kanamycin concentrations upto 50µg ml^{-1}, whereas transformants with pBBR122 were able to resist chloramphenicol at a concentration of 10µg ml^{-1}. The optimum electric field strength necessary for maximum transformation efficiency of bacterial cells ranges from 2 to 18 kV cm^{-1} [9, 10]. It was observed that the electric strength of 15kVcm^{-1} for 5 ms gave maximum number of transformants (Table1). A total of 576 transformed colonies from three independent transformation reactions were obtained using 20 ng of transposome per reaction. The transformation efficiency was 0.6×10^6 cfu μg^{-1} of transposome. The study reports development of a simple EZ::Tn5 based approach for random mutagenesis in *M. ciceri* using electroporation technique. This produced mutants at a high efficiency as compared to the conventional bi-parental or tri-parental mating techniques. Use of electroporation makes the mutant generation less cumbersome and generation of satellite colonies was less. An auxotrophic mutant MC8A2 was identified as dependent on proline by cross-pool auxanography. This mutant was further confirmed by its growth on two sets of VMM plates, one containing required nutritional requirement and another without it. The MC8A2 mutant was unable to grow without proline whereas growth in VMM with proline or YEMA was similar to that of the wild type. This shows that biosynthesis of proline was affected in the mutant and thus was auxotrophic for proline.

A 800 bp long amplicon was obtained using chromosomal DNA of the mutants as template in PCR reaction with primers specific to the Tn5 whereas no amplification was observed in wild type *M. ciceri* Ca181 (fig.1). Sequencing of the PCR product confirmed the presence of Tn5 within the chromosomal DNA of the transformant. An IPCR product of ~1 kb length was obtained (fig. 2) and sequenced to identify the genomic insertion site of Tn5 transposon in the mutant MC8A2. Pyrroline-5-carboxylate reductase gene was found to be disrupted by transposon in the mutant MC8A2, which suggested that the gene is involved in the proline biosynthesis pathway [11].

Concluding Remarks

The present study is the first report of successful transformation of a *Mesorhizobium* with transposome complex which enabled the rapid generation of large numbers of random Tn5 transposon mutants. All screened Tn5 mutants carried only a single transposon insertion and were stable over successive culturing. The current study demonstrated the utility of

Tn5 random mutagenesis system by identifying proline auxotroph mutant. This *M. ciceri* Ca181 transposon mutant library has already been successfully screened for several traits in our laboratory for identification of genes for salt and heat tolerance (our unpublished results). The advantage of Tn5 mutagenesis with the transposome technology over the standard *in vivo* transposase expression approach are the high efficiency and the lack of requirement for complex replicative vectors or native inducible promoters to drive transposase expression. Transposome electroporation is likely to become the method of choice for generating transposon insertion libraries.

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APPENDICIES

Table 1: Effect of Electric Field Strength for 5ms on the Electro transformation of *M. ciceri* Ca181 with Broad Host Range Plasmid Vector pBBR122

Field Strength (Kvcm^{-1})	Total Trans Formants 10^8
12	0.6
13	2.0
14	2.5
15	3.4
16	2.8
17	0.8
18	0.6

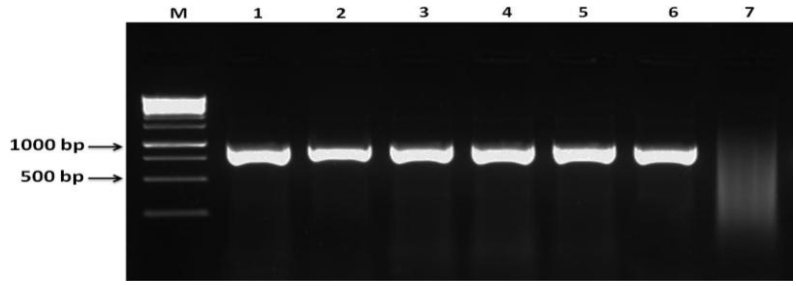

Figure 1: Amplification of the DNA Sequences with Tn5 Transposome Insertion in the Chromosomal DNA of *M. Ciceri* Ca181 the PCR Products (800 bp) Amplified from Five Randomly Chosen Tn5 Mutants (Lanes 1-6), the Wild Type (Lane 7). A 1 Kb DNA Ladder (Fermentas) was Used as a Standard Marker (M)

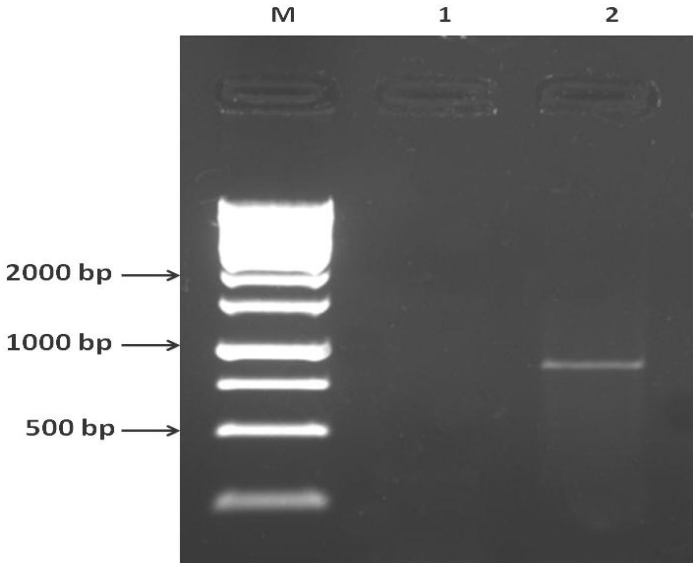

Figure 2: Gel Electrophoresis of IPCR Product of ~1 Kb Length the PCR Products Amplified From the Wild Type (Lane 1) and MC8A2 (Tn5 Mutant) (Lane 2). A 1Kb DNA Ladder (Fermentas) was Used as a Standard Marker (M)